NUTRITIONAL GROWTH REQUIREMENTS FOR SUBMERGED CULTURES OF THE ECTOMYCORRHIZAL FUNGI *CENOCOCCUM GEOPHILUM* Fr.

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ABSTRACT - The capacity of *Cenococcum geophilum* to produce considerable biomass of submerged mycelial condensations in ten different culture media was tested. The best production was obtained with glucose ammonium tartrate (mycelium dry weight 4.52 mg ml), glucose ammonium chloride (4.05 mg ml) and Macaya-Lizano medium (3.72 mg ml). After testing different sources of hydrocarbons and of inorganic (NO3-, NH4+) and organic N (amino acids and undefined substances), a new optimized medium was developped resulting in a significant increase of biomass production (5.96 mg ml).

RÉSUMÉ - Dix types de milieux de culture ont été testés afin d'améliorer, en culture submergée, la production de mycélium de *Cenococcum geophilum*. Le meilleur rendement a été obtenu avec les milieux suivants: glucose / tartrate d'ammonium (poids sec du mycélium: 4,52 mg ml), glucose / chlorure d'ammonium (4,05 mg ml) et Macaya-Lizano (3,72 mg ml). Après avoir testé différentes sources de carbone, d'azote inorganique (NO3-, NH4+) et d'azote organique (acides aminés et substrats complexes) un nouveau milieu a été développé; il permet un accoissement significatif de la production de biomasse.

INTRODUCTION

The role of ectomycorrhizal fungi in the growth of woody plant species is of significant agricultural and ecological importance, and it is well established that many trees need an ectomycorrhizal association (Mauperin et al., 1987). Water and nutrient uptake from the surrounding ecosystem are considerably improved through the soil mycelium of the mycorrhizal fungi, due to increase in surface and absorption efficiency. Soil or seedlings inoculation with ectomycorrhizal fungi can render the introduction of tree species possible in areas which otherwise would not support their growth (Harvey, 1991). The first step in an artificial inoculation program requires the knowledge of the nutritional requirements of the fungus and the *in vitro* growth-optimization to produce suitable, low cost inocula.

Cenococcum geophilum has been frequently described in ectomycorrhizal association with economically important tree families: Myrtaceae, Salicaceae and Pinaceae (Trappe, 1962; Chilvers, 1968; Heslin & Douglas, 1986; Danielson & Pruden, 1989)

In spite of its ecological importance, very little information is available regarding the nutritional factors affecting its growth and biomass production in submerged culture.

In the present study we tested the capacity of *Cenococcum geophilum* to producing considerable biomass of submerged mycelial condensations in a whole range of different culture media. We can thus propose an optimized medium with the final objective of producing sufficient quantity of inocula for large-scale forest application.

MATERIALS AND METHODS

1. Strain

Experiments were done with strain X01-29 (collected in Birmensdorf, Zurich) supplied by Vitroculture SA., Porrentruy, Switzerland.

2. Preparation of mycelial inoculum

Shaken cultures of *Cenococcum geophilum* were grown in glucose ammonium chloride medium (pH 4.7) at 25 °C and 250 rpm. After an incubation period of 28 days, the mycelial condensations were aseptically homogenized and used as inoculum for all the experiments.

3. Biomass growth in different culture media

Cenococcum geophilum was grown in submerged cultures in the following media:

1- Molasses (molasses 30g),
2- Malt extract (malt extract 15g),
3- Modified Lutz (malt extract 10 g, NH_4NO_3 1g, $(NH_4)_2PO_4$ 1g, $MgSO_4.7H_2O$ 0.025g),
4- Glucose alanine (glucose 10g, DL-alanine 1g, KH_2PO_4 2g, $MgSO_4.7H_2O$ 0.2g),
5- Glucose peptone yeast (glucose 10g, peptone 10g, yeast extract 10g),
6- Melin-Norkrans' modified medium (KH_2PO_4 0,5g, $CaCl_2$ 0.05g, NaCl 0.025g, $(NH_4)2HPO_4$ 0.25g, $MgSO_4.7H_2O$ 0.15g, 1% solution of $FeCl_3$ 1.2ml, malt extract 3g, sucrose 20g),
7- Yeast malt extract (yeast extract 3g, malt extract 3g, peptone 5g, glucose 10g),
8- Macaya-Lizano (KH_2PO_4 1,5g, $ZnSO_4.7H_2O$ 0.2g, $(NH_4)_2SO_4$ 1g, $MgSO_4.7H_2O$ 0.2g, $CaCl_2$ 0.4g, L-asparagine 1.12g, DL-serine 2g),
9- Glucose ammonium chloride (KH_2PO_4 0.5g, $MgSO_4.7H_2O$ 0.5g, NH_4Cl 0.5g, glucose 10g, malt extract 5g, 1% solution of ferric citrate 0.5ml, glucose 10g),
10- Glucose ammonium tartrate (KH_2PO_4 1g, $MgSO_4.7H_2O$ 0.5g, ammonium tartrate 1g, $ZnCO_4$ (1:500 aqueous solution) 0.5ml, glucose 30g, 1% solution of ferric citrate 0.5ml).

The compounds were dissolved in 1000 ml distilled H_2O. The pH of the media was adjusted to 5.0 with either HCl or NaOH before autoclaving, and after autoclaving 1.0 mg of biotin and 2.0 mg of thiamine were added to each medium tested.

For each medium tested, three 500 ml flasks containing 330 ml of sterile medium were inoculated with 10 g of freshly homogenized mycelia (0.3g dried weight). The flasks were incubated at 25°C for 21 days and flushed with 3 l/h sterile air. At the end of the incubation period the mycelial condensations were

washed and dried. The biomass was expressed as mg dry weight/ml culture medium.

4. Optimization of the medium

Cenococcum geophilum was grown in glucose ammonium tartrate standard medium modified as follows:

- carbohydrates: 8 different carbohydrates (2% w/v) were tested in a glucose-free medium (Table I)

- inorganic nitrogen: ammonium tartrate was substituted with 6 different sources of inorganic nitrogen with the same N-content (84 mg l, Table I)

- organic nitrogen: 5 amino acids (10 mM) or 1 g l complex substrates (casein hydrolysate (Fluka) or soybean meal peptone (Fluka)) were also tested (Table I).

For each modification, three tests were made in the same conditions as above and the biomass was measured.

The carbohydrate, inorganic and organic nitrogen source supporting maximum mycelial growth were chosen for further nutritional study. Different concentrations of these components were tested to determine the optimum level required for growth. Finally the effect of 1-2.5 ml l of commercial safflower oil and soybean oil in the optimized medium was measured as before.

All the solutions used were autoclaved 20 minutes at 121°C, except vitamins that were sterilized by Millipore filtration (0.2μM membrane) and added to the cooled medium.

Culture medium	mycelia (mg dry weight/ml)
Molasses	1.58 ± 0.19
Malt extract	2.03 ± 0.22
Modified Lutz	2.31 ± 0.25
Glucose alanine	2.69 ± 0.23
Glucose peptone yeast	3.07 ± 0.27
Melin-Norkrans' modified medium	3.14 ± 0.29
Yeast malt-extract peptone	3.46 ± 0.30
Macaya-Lizano	3.72 ± 0.27
Glucose ammonium chloride	4.05 ± 0.31
Glucose ammonium tartrate	4.52 ± 0.29

Table I. - Dry weight of mycelia of *Cenococcum geophilum* (mean of 3 replicates) in the different culture media.

Tableau I. - Poids sec du mycélium de *Cenococcum geophilum* (moyenne de 3 expériences) sur les différents milieux.

RESULTS

1. Biomass production in different culture media

Table I shows the yields obtained in the 10 media tested. Glucose ammonium tartrate medium was chosen for further optimization.

2. Optimization of the culture medium

Of the 8 carbon sources tested, glucose and fructose gave maximum mycelial growth. No growth occurred with arabinose and cellulose, ribose, starch and cellobiose were unsuitable (Table II).

Among the inorganic sources of nitrogen, maximum growth was observed with di-ammonium tartrate, and good growth was also observed with ammonium chloride, ammonium nitrate and ammonium tartrate. No growth occurred with ammonium acetate or sodium nitrite. The yield was increased by addition of casein hydrolysate or peptone in the glucose ammonium tartrate medium (Table II).

Different concentrations of glucose, casein and di-ammonium tartrate were tested (fig. 1). From these results, the following optimized medium was designed: glucose 20 g, di-ammonium tartrate 0.25g, casein hydrolysate 1.5g, biotin 1mg, thiamine 2mg, KH_2PO_4 1g, $MgSO_4.7H_2O$ 0.5g, $ZnCO_4$ (1:500 aqueous solution) 0.5 ml and 1% solution of ferric citrate 0.5ml, giving a yield of 5.96 ± 0.33 mg dry weight ml.

No significant yield increase was obtained with the addition of commercial safflower oil (0.5, 1.0, 1.5, 2.0, 2.5ml) or commercial soybean oil (0.5, 1.0, 1.5, 2.0, 2.5 ml) to the optimized medium. Figure 2 compares the growth curves of *Cenococcum geophilum* (28 days, 25°C) in two standard media for ectomycorrhizal fungi: Melin-Norkrans' modified medium (MMN) and glucose ammonium tartrate medium (GAT) with our optimized medium (Gd-ATC: glucose di-ammonium tartrate casein medium).

DISCUSSION

The carbon requirements of *Cenoccocum geophilum* are similar to those of other mycorrhizal fungi (Palmer & Hacskaylo, 1970; Straatsma & Van Griensven, 1986). Glucose was the best carbon source for mycelial growth and this agrees with the general concept that, among all the hexoses, glucose is biologically the most effective energy source.

The failure of *Cenococcum geophilum* to grow with sodium nitrite resembles a common situation in fungi (Song et al., 1987), calcium nitrate produces low yields, probably due to precipitation with phosphates and N in the ammonium form is generally suitable for the fungi development. Melin & Mikola (1948) compared ammonium, casein hydrolysate and the individual amino acids leucine, proline, valine, histidine and arginine as N source for *Cenoccocum geophilum*. They showed that of all the amino acids tested, only arginine induced yields comparable with those obtained with ammonium, whereas casein hydrolysate sustained the best yield. Laiho (1970) using a series of strains of *Paxillus involutus* also compared growth performance on ammonium with those of several amino acids, and reported that only glutamic acid and arginine gave yields as high as those on ammonium. Our results also show (Table II) a good yield of the fungus on L-arginine and L-asparagine,

carbohydrates 2%	mg/ml (dry weight)	Inorganic nitrogen (84 mg)	mg/ml (dry weight)	Organic nitrogen (10 mM)	mg/ml (dry weight)
L (+) Arabinose	no growth	ammonium acetate	no growth	L-Proline	4.45 ± 0.26
Cellulose	2.53 ± 0.19	sodium nitrite	no growth	L-Glutamic acid	4.52 ± 0.25
D (-) Ribose	3.02 ± 0.23	calcium nitrate	1.91 ± 0.28	L-Alanine	4.56 ± 0.27
Starch	2.17 ± 0.21	ammonium nitrate	4.50 ± 0.31	L-Asparagine	4.97 ± 0.28
D (+) Cellobiose	2.86 ± 0.19	ammonium chloride	4.65 ± 0.32	L-Arginine	5.05 ± 0.32
D (+) Sucrose	3.66 ± 0.31	di-ammonium tartrate	4.91 ± 0.29	peptone 1g	5.12 ± 0.29
D (+) Trehalose	4.03 ± 0.25			casein hydrolysate 1g	5.43 ± 0.27
D (-) Fructose	4.39 ± 0.29				

Table II. - Mycelial growth (mg dry weight ml) of *Cenococcum geophilum* in glucose di-ammonium tartrate medium with different sources of carbon and nitrogen.

Tableau II. - Croissance du mycélium de *Cenococcum geophilum* (mg poids sec ml) dans le milieu de glucose di-ammonium tartrate avec différentes sources de carbone et d'azote.

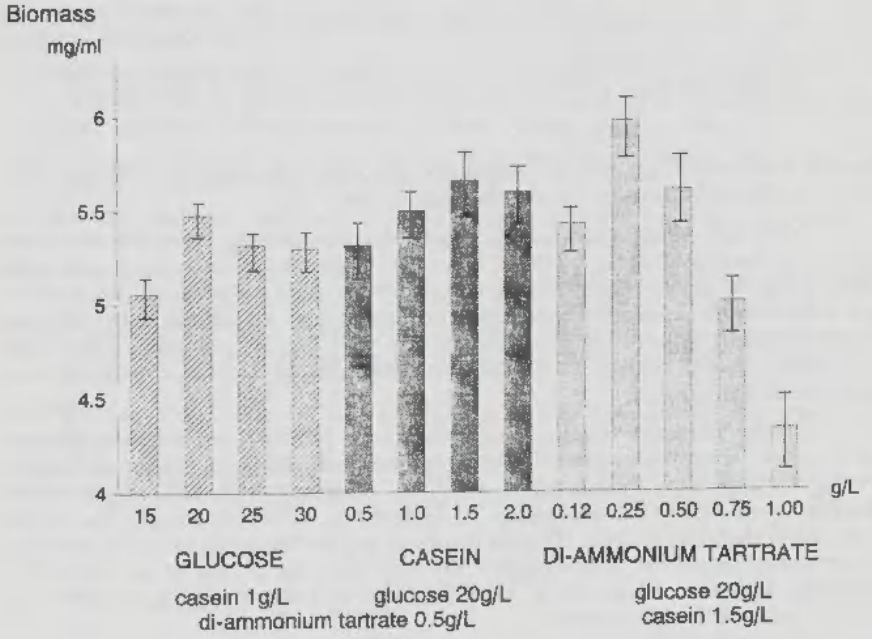

Fig.1: Biomass of mycelia (mg dry weight ml) obtained after 21 days, in media with different concentrations of glucose, casein hydrolysate or di-ammonium tartrate.

Fig.1: Biomasse du mycélium (mg poids sec ml) produite après 21 jours de croissance dans un milieu avec différentes concentrations de glucose, d'hydrolysat de caséine ou de di-ammonium tartrate.

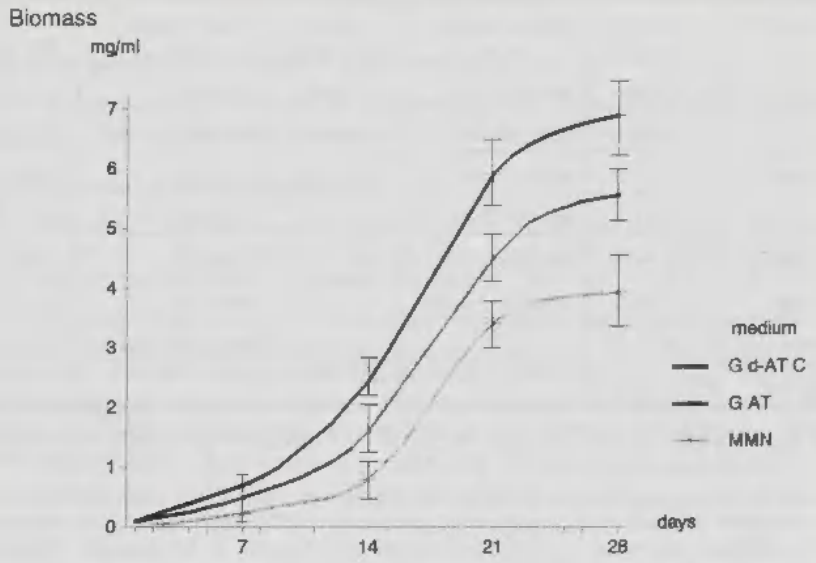

Fig. 2: Growth curves of *Cenococcum geophilum* in two standard media for ectomycorrhizal fungi (MMN and GAT) and in the Gd-ATC optimized medium.

Fig. 2: Courbes de croissance de *Cenococcum geophilum* dans deux milieux standard pour champignons ectomycorrhiziques (MMN et GAT) et dans le milieu Gd-ATC.

however the optimal growth of submerged mycelia, was supported by the additions of complex substrates (ie. casein hydrolysate).

Although several reports indicate the stimulating effect of materials containing fatty acid on growth of ectomycorrhizal fungi, no significant yield increase was observed when commercial safflower oil or commercial soybean oil were added to the optimized medium. This failure may be related to the absence of lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) which release free fatty acids from several sources that has been observed in *Cenoccocum geophilum* (Caldwell et al., 1991) .

Of the medium tested, Melin-Norkrans, Glucose ammonium chloride and Glucose ammonium tartrate were originally developped for the culture of ectomycorrhizal fungi, and often used in studies of growth requirements (Hacskaylo et al., 1965; Straatsma & Griensven, 1986; Torres & Honrubia, 1991). We showed (Table II) that sucrose supported only a poor yield of *Cenoccocum geophilum*; this explains the low biomass produced in the Melin-Norkrans' medium. However, a suitable yield of *Cenoccocum geophilum* was obtained with both other media.

The optimization reported here allowed yield increase in mycelial biomass of 30 % due to the addition of an organic N source and to the establishment of optimal concentrations for the main C and N sources. Contrary to the general idea that ectomycorrhizal fungi grow very slowly in liquid culture (Chapman et al., 1990) our results show that a correct optimization of the culture medium allows fast growth of considerable biomass in submerged cultures.

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LITERATURE

CALDWELL B.A., CASTELLANO M.A. and GRIFFITHS R.P., 1991 - Fatty acid esterase production by ectomycorrhizal fungi. *Mycologia* 83: 233-236.

CHAPMAN W.K., BERCH S.M. and BALLARD T.M., 1990 - *In vitro* growth of ectomycorrhizal fungi on dilute agar. *Mycologia* 82: 526-527.

CHILVERS G.A., 1968 - Some distinctive types of eucalypt mycorrhizae. *Austral. J. Bot.* 16: 49-70.

DANIELSON R.M. and PRUDEN M., 1989 - The ectomycorrhizal status of urbans Spruce. *Mycologia* 81: 335-341.

HACSKAYLO E., PALMER P.J. and VOZZO J.A., 1965 - Effect of temperature on growth and respiration of ectotrophic mycorrhizal fungi. *Mycologia* 57: 748-756.

HARVEY L.M., 1991 - Cultivation techniques for the production of ectomycorrhizal fungi. *Biotechnol. Adv.* 9: 13-29.

HESLIN M.C. and DOUGLAS G.C., 1986 - Synthesis of Poplar mycorrhizas. *Trans. Brit. Mycol. Soc.* 86: 117-122.

LAIHO O., 1970 - *Paxillus involutus* as a mycorrhizal symbiont of forest trees. *Acta Forest. Fenn.* 106: 1-65.

MAUPERIN C., MORTIER F., GARBAYE J., LE TACON F. and CARR G., 1987 - Viability of an ectomycorrhizal inoculum produced in a liquid medium and entrapped in a calcium alginate gel. *Canad. J. Bot.* 65: 2326-2329.

MELIN E. and MIKOLA P., 1948 - Effects of some amino acids on the growth of *Cenococcum graniforme*. *Physiol. Pl.* 1: 109-112.

PALMER J.G. and HACSKAYLO E., 1970 - Ectomycorrhizal fungi in pure culture. I. Growth on single carbon sources. *Physiol. Pl.* 23: 1187-1197.

SONG C.H., CHO K.Y. and NAIR N.G., 1987 - A synthetic medium for the production of submerged cultures of *Lentinus edodes*. *Mycologia* 79: 866-876.

STRAATSMA G. and VAN GRIENSVEN L.J.L.D., 1986 - Growth requirements of mycelial cultures of the mycorrhizal mushroom *Cantharellus cibarius*. *Trans. Brit. Mycol. Soc.* 87: 135-141.

TRAPPE J.M., 1962 - Fungus associates of ectotrophic mycorrhizae. *Bot. Rev.* 28: 538-606.

TORRES P. y HONRUBIA M., 1991 - Dinamica de crecimiento y caracterizacion de algunos hongos ectomicorricios en cultivo. *Cryptogamie, Mycol.* 12: 183-192.